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ORIGINAL ARTICLE

The role of the N-terminal caspase cleavage site in the nucleoprotein of influenza A virus in vitro and in vivo

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Abstract The N-terminal caspase cleavage in the nucleoprotein (NP) of influenza A virus is correlated with the host origin of the virus, thus could be a molecular determinant for host range. We studied how mutations targeting the NP cleavage motif of human and avian influenza viruses affect virus replication in vitro and in vivo. The "avian-like" D_{16}→G substitution in the NP, which makes this protein resistant to cleavage, did not significantly affect the human A/Puerto Rico/8/34 (H1N1) virus replication in vitro but decreased the lethality of this virus in mice by 68-fold. Gene incompatibility contributed to the attenuated phenotype of the reassortant A/Puerto Rico/8/34 virus with avian NP derived from A/Teal/Hong Kong/w312/97 (H6N1) virus in vitro and in vivo. Insertion of the "human-like" G_{16}→D mutation into avian NP, which resulted in susceptibility to caspase cleavage, did not rescue virulence, but made the reassortant virus even more attenuated. Introducing the human-like G_{16}→D substitution into the NP of highly pathogenic A/Vietnam/1203/04 (H5N1) virus decreased lethality in mice. We confirmed that position 16, which associated with the N-terminal caspase cleavage of the NP, is important for optimal virus fitness in vitro and in vivo. An avian-like mutation at position 16 in the NP of human virus as well as a human-like substitution at this residue in avian NP both resulted in virus attenuation.

Introduction

Separate genetic lineages of influenza A viruses have been established in birds, humans, horses, and pigs [23, 24]. The genes and viral proteins responsible for host range restriction as well as host species barriers for influenza A viruses have been characterized [11, 13]. The experimental data suggest that the gene encoding the nucleoprotein (NP) is one of the determinants of host specificity of influenza A viruses [1, 19, 21]. Multiple functions of NP have been well studied (reviewed by Portela and Digard [16] and [2]): NP functions primarily in replication and packaging by regulating transcription and by forming the ribonucleoprotein (RNP) to encapsidate the virus genome. In addition, NP interacts with two subunits of the viral RNA-dependent RNA polymerase and the viral matrix protein and also interacts with cellular polypeptides, including actin, components of the nuclear import and export apparatus, and a nuclear RNA helicase. NP is considered to serve as a key adaptor molecule between viral and host cell processes.

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Phylogenetic analysis has discriminated NP genes of influenza A viruses into at least two classes. Each class is characteristic for the strains that originated from human or nonhuman influenza viruses [3, 5, 7]. In infected cells, cleavage of influenza A virus NP, which has a molecular weight of 56 kDa (NP56), creates a form with a molecular weight of 53 kDa (NP53) [28]. The intracellular cleavage of NP is correlated with the host origin of the virus. Only NPs of human influenza virus strains are sensitive to host proteases and cleaved in infected cells, whereas those of the avian strains are resistant to intracellular proteases and fail to be cleaved [29, 30]. The NP of human influenza viruses is cleaved at the N-terminal amino acid motif ($ETD_{16}{}^{\downarrow}G$) that is characteristically recognized by caspases, which play a key role in apoptosis; NPs that originate from avian strains contain the motif $ETG_{16}G$ and therefore are resistant to cleavage [31]. The roles of these modifications are not well studied yet; however, as only uncleaved NP56 is incorporated into virions, it was proposed that intracellular cleavage prevents incorporation of the viral RNP into the virus [28, 31]. A recombinant human influenza A/WSN/33 (H1N1) virus that contained NP with an "avian-like" mutation, $D_{16}\rightarrow G$, replicated more slowly than did the wild-type virus; the mutated NP was resistant to cleavage by cellular caspases [32]. However, the mechanism of how the mutations at the caspase cleavage motif affect the pathogenicity of human and avian influenza viruses in animal models was not investigated at that time.

Because the only NP that is cleaved in infected cells is that of human influenza A viruses and influenza B viruses, which are also mainly isolated from humans (influenza B virus has been isolated only once from another species—seals) [14, 29, 31], it is reasonable to propose that this feature of influenza A virus NP is related to host specificity and/or to pathogenicity. To test this hypothesis in a mammalian model, we first created by using reverse-genetic techniques a recombinant human A/Puerto Rico/8/34 (H1N1) (PR/8) virus with an NP that contained the avian-like $D_{16}\rightarrow G$ mutation (PR/8 $NP_{D16\rightarrow G}$) and two reassortant viruses in which the viral genome of PR/8 contained the avian-originated NP derived from A/Teal/Hong Kong/w312/97 (H6N1) virus (designated as PR/8 × NP Teal/HK) or an avian-originated NP containing a "human-like" motif ($G_{16}\rightarrow D$) at the caspase cleavage site (designated as PR/8 × $NP_{G16\rightarrow D}$ Teal/HK virus) (Fig. 1a). The pathogenicity and replication of these viruses were characterized in a mouse model. A recombinant of a highly pathogenic avian H5N1 virus isolated from humans (A/Vietnam/1203/04 [VN/04]) and an H5N1 virus that contained a $G_{16}\rightarrow D$ mutation in its NP (VN/04 $NP_{G16\rightarrow D}$) were also created (Fig. 1a). The pathogenicity of these viruses was studied in mice and chickens.

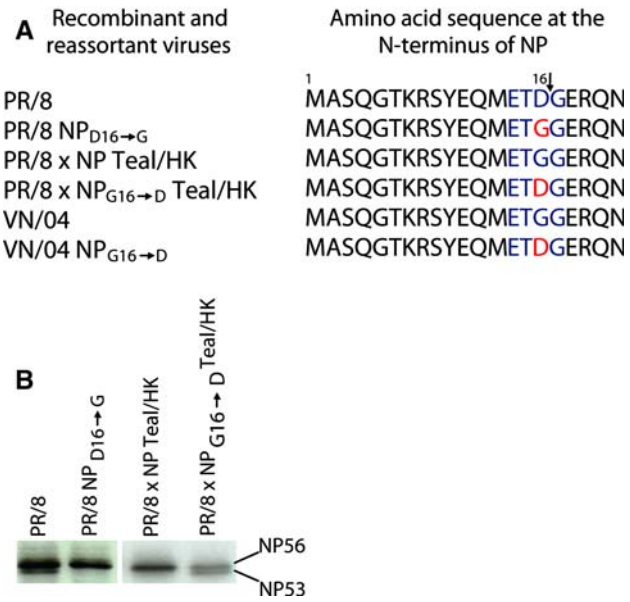

Fig. 1 **a** Amino acid sequences of the N-terminus of NP of the recombinant and reassortant viruses. **b** Cleavage of NP in virus-infected cells. MDCK cells were infected with viruses at an MOI of approximately 10 PFU/cell, and proteins were labeled with [^{35}S]-methionine 5–6.5 h after infection. Twenty hours after infection, cells were harvested, lysed in a sample buffer, and subjected to electrophoresis in a 12.5% polyacrylamide gel under reducing conditions. The gel was dried and exposed to X-ray film to obtain images

Materials and methods

Viruses and cells

The laboratory human virus strain A/Puerto Rico/8/34 (H1N1) (PR/8) and the avian strain of H6N1 subtype A/Teal/Hong Kong/w312/97 (Teal/HK) were obtained from the repository of the Division of Virology, Department of Infectious Diseases, St Jude Children's Research Hospital; a human isolate of the highly pathogenic avian H5N1 virus A/Vietnam/1203/04 (VN/04) was obtained from World Health Organization—collaborating laboratories in Asia. Viruses were grown in 10-day-old embryonated chicken eggs, and virus-containing allantoic fluids were used in experiments. Experiments with the VN/04 virus were conducted in a biosafety level 3+ containment facility.

Madin-Darby canine kidney (MDCK) and 293T human embryonic kidney cells were obtained from the American Type Culture Collection (Manassas, VA). MDCK cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum. 293T cells were cultured in Opti-MEM I (Life Technologies) that contained 5% fetal bovine serum.

Sequence analysis

Viral RNA was isolated directly from virus-containing allantoic fluid by using an RNA isolation kit (RNeasy; Qiagen). The universal primer set for influenza A virus was used for RT-PCR [9]. The Hartwell Center for Bioinformatics and Biotechnology at St Jude Children's Research Hospital determined the DNA template sequence by using Big Dye Terminator (v.3) chemistry and synthetic oligonucleotides. Samples were analyzed on 3700 DNA analyzers (Applied Biosystems).

Generation of recombinant viruses and site-directed mutagenesis

The eight genes of the PR/8 and VN/04 viruses and the NP gene of Teal/HK were described previously [8, 10, 18]. Plasmids were sequenced as described in the preceding text, and the sequences were compared with those generated from the wild-type virus. Only clones whose sequence exactly matched the parental virus sequence were used for virus rescue by reverse genetics. Viruses were rescued by using the eight-plasmid system [8] with minor modifications. Briefly, eight plasmids (1 μg of each) were incubated for 45 min with Trans-LTI (Panvera) in Opti-MEM I and were used to transfect 293T cells. Supernatant collected from transfected cells after 72 h was used to inoculate 10-day-old embryonated chicken eggs. Allantoic fluid that contained virus was harvested, and its infectivity was titrated in eggs; virus titers were expressed as $\log_{10}$ of the 50% egg infective dose per 0.1 ml of fluid ($\log_{10}$ EID_{50} per 0.1 ml), according to the method of Reed and Muench [17]. Virus stocks were divided into aliquots and stored at −80°C.

Point mutations that encoded D_{16}→G and G_{16}→D substitutions (Fig. 1a) were inserted into the NP gene during PCR by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and a set of NP-specific primers. Viruses were rescued as described above, and the NP genes were sequenced to confirm the presence of the mutations.

Labeling of proteins in virus-infected cells with [^{35}S]-methionine and electrophoresis

Confluent monolayers of MDCK cells were infected with viruses at a multiplicity of infection (MOI) of approximately ten PFU/cell. After incubation, the cells were washed twice with phosphate-buffered saline (PBS), and MEM supplemented with 0.3% bovine serum albumin was added to the wells. Five hours after infection, the medium was removed, the cells were washed twice with MEM without methionine, and [^{35}S]-methionine (TRAN^{35}S-LABEL, MP Biomedicals) was added (15 μCi/culture) for 1.5 h. After that, the medium with the radiolabel was removed, and the cells were washed twice with PBS and incubated in MEM with 0.3% bovine serum albumin. Twenty hours after infection, the medium was removed, and the cells were washed with PBS, harvested, lysed in a sample buffer, and subjected to electrophoresis in a 12.5% polyacrylamide gel under reducing conditions. The gel was dried and exposed to X-ray film (Kodak Biomax).

Replication kinetics assay

The replication kinetics of the viruses was determined in MDCK cells as described previously [27]. Briefly, to determine single-step growth curves, we infected cells with viruses at an MOI of approximately 2.5 PFU/cell. After incubation, the cells were washed, and infection medium was added to the wells. Supernatants were collected 2, 4, 6, 8, and 10 h after infection and stored at −70°C before titration. To determine multistep growth curves, we infected cells with viruses at an MOI of approximately 0.01 PFU/cell. Supernatants were collected 12, 24, 36, 48, 60, and 72 h after infection and stored at −70°C before titration. Viral titers in harvested supernatants were determined in previously described plaque assays in which MDCK cells were used [27].

Pathogenicity and replication in mice

Six-week-old female BALB/c mice (The Jackson Laboratory) were used to determine the mouse 50% lethal dose (MLD_{50}) and to investigate viral replication and pathogenesis. To determine the MLD_{50}, mice in groups of four were infected intranasally as described previously [12]. MLD_{50} was calculated by the method of Reed and Muench [17] and was defined as the number of EID_{50} resulting in 50% mortality. Viral pathogenicity (organ distribution and titers) was studied in mice inoculated as described earlier [12] with 50 μl PBS-diluted allantoic fluid that contained approximately 0.5×10^3 EID_{50} of virus. Three mice in each virus group were killed on days 1, 3, 5, 7, 9, and 11 after inoculation, and the lungs, brains and spleen of each mouse were removed. A homogenate of each tissue (approximately 10% [*w*/*v*]) was prepared in PBS and titrated in 10-day-old embryonated chicken eggs to determine EID_{50}. The lower limit of virus detection was 0.5 $\log_{10}$ EID_{50} per 0.1 ml of tissue homogenate.

Pathogenicity test in chickens

The intravenous virus pathogenicity index (IVPI) of VN/04 and VN/04 $NP_{G16→D}$ viruses was determined as described

by Capua and Mutinelli [4] with slight modifications. Infective allantoic fluid was diluted with PBS to obtain the infective dose ($10^{4.25}$ EID_{50}), and 0.1 ml was injected intravenously into each of ten 6-week-old, specific pathogen-free chickens. The chickens were examined two times a day for clinical signs of disease. Pathogenicity was scored as 0 (no signs of illness), 1 (signs of illness), 2 (signs of severe illness), or 3 (death within 24 h of inoculation).

Results and discussion

Generation of the recombinant viruses and characterization of the N-terminal NP cleavage

Recombinant and reassortant viruses that contained mutations at residue 16 of NP were successfully rescued by using an 8-plasmid reverse genetics system for influenza A viruses [8]. The identity of the generated viruses and the amino acids at the NP N-terminus was confirmed by sequencing (Fig. 1a). By using MDCK cells, we evaluated the cleavability of NP of the recombinant viruses PR/8 and PR/8 $NP_{D16\rightarrow G}$ and of the reassortant viruses PR/8 × NP Teal/HK and PR/8 × $NP_{G16\rightarrow D}$ Teal/HK. Polyacrylamide gel electrophoresis (PAGE) of virus-specific proteins labeled with [^{35}S]-methionine revealed that NP of the human virus was cleaved in infected cells and was represented by two forms, NP56 and NP53, whereas NP that contained the avian-like $D_{16}\rightarrow G$ mutation was represented only by the NP56 form, i.e. the mutant NP was not cleaved (Fig. 1b). The NP that originated from avian virus was represented only by the uncleaved NP56 form. In contrast, the human-like substitution $G_{16}\rightarrow D$ made avian NP susceptible to proteolysis in infected cells: two forms of NP of PR/8 × $NP_{G16\rightarrow D}$ Teal/HK reassortant virus were observed in polyacrylamide gels (Fig. 1b). These results support the previous observation of Zhirnov et al. [32] that D_{16} is essential for the N-terminal cleavage of NP of human influenza A virus in infected cells. In addition, we showed that the substitution $G_{16}\rightarrow D$ in NP that originated from an avian influenza virus made this protein cleavable in infected cells. The cleavability of NP of recombinant PR/8 and PR/8 $NP_{D16\rightarrow G}$ viruses was also characterized in cultures of primary chicken embryo fibroblasts (data not shown). The results were similar to those observed in MDCK cells: the cleavability of NP of human influenza A virus is determined by the presence of D at position 16, and this feature of NP is not dependent on the species from which the infected cells originated.

Replication kinetics of the recombinant viruses in vitro

The growth capacities and replication kinetics of recombinant human viruses and human-avian NP reassortants were characterized in MDCK cells under the conditions of single-step and multistep growth cycles. PR/8 and PR/8 $NP_{D16\rightarrow G}$ viruses had similar single-step growth curves (Fig. 2a). The titers of reassortant viruses in which the avian NP was inserted in the genetic backbone of the human virus were about 1.0 $\log_{10}$ lower than those of the PR/8 virus; the differences in titers between PR/8 × NP Teal/HK and PR/8 × $NP_{G16\rightarrow D}$ Teal/HK observed in a single-step growth cycle were almost negligible (Fig. 2a). In a multistep growth experiment, titers of PR/8 virus with the avian-like $D_{16}\rightarrow G$ mutation in its NP were 0.5–1.0 $\log_{10}$ lower than those of the wild-type virus; PR/8 × NP Teal/HK reassortant virus reached titers about 1.0 $\log_{10}$ lower than those of the PR/8 $NP_{D16\rightarrow G}$ virus, and the attenuation of the PR/8 × $NP_{G16\rightarrow D}$ Teal/HK virus was even greater (Fig. 2b). However, these differences were not statistically significant and, overall, no significant differences were observed among pairs of viruses in either single- or multistep replication. Sequencing of the NP

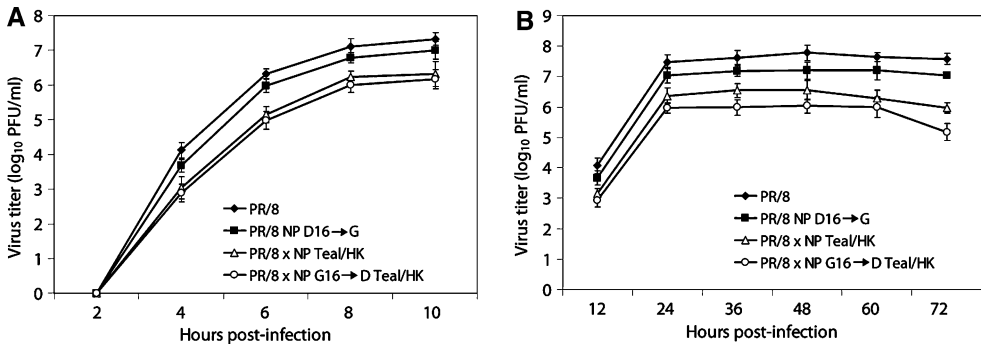

Fig. 2 Replication kinetics of recombinant human viruses and human-avian NP reassortants in MDCK cells. **a** Single-step growth curve. Cells were infected with virus at an MOI of approximately 2.5 PFU/cell. **b** Multistep growth curve. Cells were infected with virus at an MOI of approximately 0.01 PFU/cell. *Error bars* represent the standard deviation (*SD*) of the mean. Data from three experiments are presented

genes of viruses harvested 72 h after infection showed that the sequences were identical to those of the infecting viruses, i.e., no reverse mutations were revealed.

Pathogenicity of the recombinant viruses in vivo

To determine how the mutations within the caspase cleavage site of NP influence the pathogenicity and replication of recombinant human influenza A viruses and human-avian NP reassortants in vivo, we studied the effects of PR/8, PR/8 $NP_{D16\rightarrow G}$, PR/8 x NP Teal/HK, and PR/8 × $NP_{G16\rightarrow D}$ Teal/HK viruses in a mouse model. The MLD_{50} values determined for the viruses demonstrated that wild-type PR/8 was highly pathogenic in mice, whereas the PR/8 $NP_{D16\rightarrow G}$ virus was about 68 times less lethal (Table 1). Reassortment of PR/8 with NP of the avian virus resulted in attenuation of pathogenicity in mice, and the insertion of the human-like caspase cleavage motif in the avian NP did not increase, but rather attenuated the pathogenicity of the human-avian NP reassortant even more: infection by PR/8 × NP Teal/HK or PR/8 × $NP_{G16\rightarrow D}$ Teal/HK reassortant virus did not result in morbidity and mortality in mice (Table 1). The replication kinetics of recombinant human viruses and human-avian NP reassortants in mouse lungs was determined. The titers of PR/8 in the lungs were highest, whereas those of PR/8 $NP_{D16\rightarrow G}$ virus were about 1.0 $\log_{10}$ lower; virus with mutated NP was cleared from the lungs at least 2 days earlier than was wild-type virus (Fig. 3). The PR/8 reassortant that contained avian NP reached titers comparable to those of the PR/8 $NP_{D16\rightarrow G}$ virus, but the clearance of this virus from the lungs was faster. The titers of the reassortant virus PR/8 × $NP_{G16\rightarrow D}$ Teal/HK in the lungs were the lowest of the viruses that were assayed; virus was detected in the lungs on days 1 and 3 only (Fig. 3). These data indicate that modification of the caspase cleavage site of the NP attenuate pathogenicity of human PR/8 virus in mice. Insertion of the mutation $D_{16}\rightarrow G$ in NP, which resulted in resistance to caspase proteolysis, reduced the pathogenicity of the virus and its replication titers and increased the clearance of the virus. The reassortment of human virus with the NP that originated from the avian influenza virus resulted in complete attenuation of viral pathogenicity. It is demonstrated that the avian, Teal/HK virus NP gene is incompatible with the genome of the human PR/8 virus. The human-like substitution inserted in the avian NP, which makes this protein susceptible to cleavage by caspases, did not rescue the virulence of the reassortant virus but increased its attenuation. Another important observation was that the differences among the viruses observed in vivo, in a mouse model are clearer and more significant than those seen in the growth kinetic experiments in which MDCK cells were used.

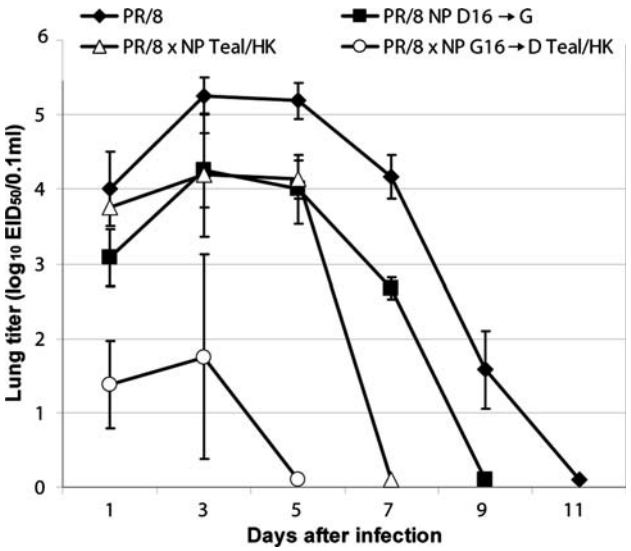

Fig. 3 Replication of recombinant human viruses and human-avian NP reassortants in mouse lungs. Six-week-old BALB/c mice were inoculated intranasally with approximately 0.5×10^3 EID_{50} of virus. Each data point represents the mean ± SD virus titer ($\log_{10}$ EID_{50}/0.1 ml of 10% tissue homogenate) from three mice. Data from two experiments are presented

Table 1 Pathogenicity of recombinant human viruses and human-avian NP reassortants in mice

Viruses	Titer in chicken eggs (log10 EID50/0.1 ml)	MLD_{50}[a]
PR/8	$10^{9.25}$	$10^{3.37}$ [b]
PR/8 $NP_{D16\rightarrow G}$	$10^{8.75}$	$10^{5.2}$ [b]
PR/8 × NP Teal/HK	$10^{7.0}$	$>10^{6.2}$
PR/8 × $NP_{G16\rightarrow D}$ Teal/HK	$10^{7.25}$	$>10^{5.95}$

[a] MLD_{50} are expressed as the number of EID_{50} that resulted in 50% mortality of infected mice. Data from three experiments are presented

[b] $P < 0.001$ according to the standard t test

Effect of the N-terminal NP cleavage site in highly pathogenic A/Vietnam/1203/04 (H5N1) virus

Our experiments showed how the mutations at residue 16 that resulted in the resistance or susceptibility of NP to cleavage by caspases influence the growth and pathogenicity of human influenza A virus and human-avian NP reassortants. To determine how the human-like substitution, which makes NP cleavable by caspases, influences the pathogenicity of a highly pathogenic avian influenza virus of the H5N1 subtype, we characterized the pathogenicity of a recombinant VN/04 virus and a variant of this virus with mutated NP, VN/04 $NP_{G16\rightarrow D}$ (Fig. 1a), in mice and chickens. The VN/04 virus is a human isolate of the highly

Table 2 Pathogenicity of recombinant avian H5N1 viruses in mice and chickens

Viruses	Titer in chicken eggs (log_{10} EID_{50}/0.1 ml)	MLD_{50}[a]	IVPI index[c]
VN/04	$10^{7.25}$	1.15[b]	3.0
VN/04 $NP_{G16\rightarrow D}$	$10^{5.25}$	24.73[b]	3.0

[a] MLD_{50} are expressed as the number of EID_{50} that resulted in 50% mortality of infected mice. Data from three experiments are presented

[b] $P < 0.001$ according to the standard t test

[c] The intravenous virus pathogenicity index (IVPI) was determined in 6-week-old chickens as described by Capua and Mutinelli [4]

pathogenic avian H5N1 virus, and it was shown previously that this virus is extremely pathogenic in mice [26].

Wild-type recombinant VN/04 virus reached higher titers in embryonated chicken eggs than did the VN/04 $NP_{G16\rightarrow D}$ variant (Table 2). The VN/04 virus was highly pathogenic and replicated systemically in mice (Table 2; Fig. 4). This virus caused disease and death at doses that are very close to the EID_{50}. Titrations to determine the MLD_{50} revealed that the variant of the VN/04 virus that had a human-like $G_{16}\rightarrow D$ mutation in its NP was about 21.5 times less lethal (Table 2). The peak of mortality in mice infected with VN/04 virus was registered on days 7 and 8 after inoculation, while most of mice infected with VN/04 $NP_{G16\rightarrow D}$ virus died on days 9 and 10 (data not shown). Inoculation with the VN/04 $NP_{G16\rightarrow D}$ virus resulted in systemic infection in mice; thus, no differences in organ tropism were observed between wild-type and mutant virus; however, the titers of the VN/04 $NP_{G16\rightarrow D}$ virus in the lungs, brain, and spleen were in general lower than those of the VN/04 virus, and the replication kinetics of the VN/04 $NP_{G16\rightarrow D}$ virus was slower (Fig. 4). Significant differences in titers between VN/04 and VN/04 $NP_{G16\rightarrow D}$ viruses were observed in the lungs and brain on day 3 ($P < 0.05$, paired t test) after virus inoculation, and spleen on days 3 and 5 post-inoculation ($P < 0.05$, paired t test). The study of pathogenicity in chickens did not reveal any differences between the VN/04 and VN/04 $NP_{G16\rightarrow D}$ viruses. Both viruses killed ten 6-week-old chickens during the 24-h period after the intravenous injection of virus (dose, $10^{4.25}$ EID_{50}), and both viruses had an intravenous virus pathogenicity index (IVPI) of 3.0 (Table 2).

These results demonstrate that the human-like $G_{16}\rightarrow D$ substitution, which makes the NP of avian origin susceptible to caspase proteolysis, attenuates the highly pathogenic avian H5N1 influenza virus in mice, but the IVPI test did not detect a significant difference in pathogenicity in chickens. It should be noted that due to the high viral dosage used for the IVPI test, it may be too limited in sensitivity to determine minor differences in pathogenicity. Because of the extremely high pathogenicity of the VN/04

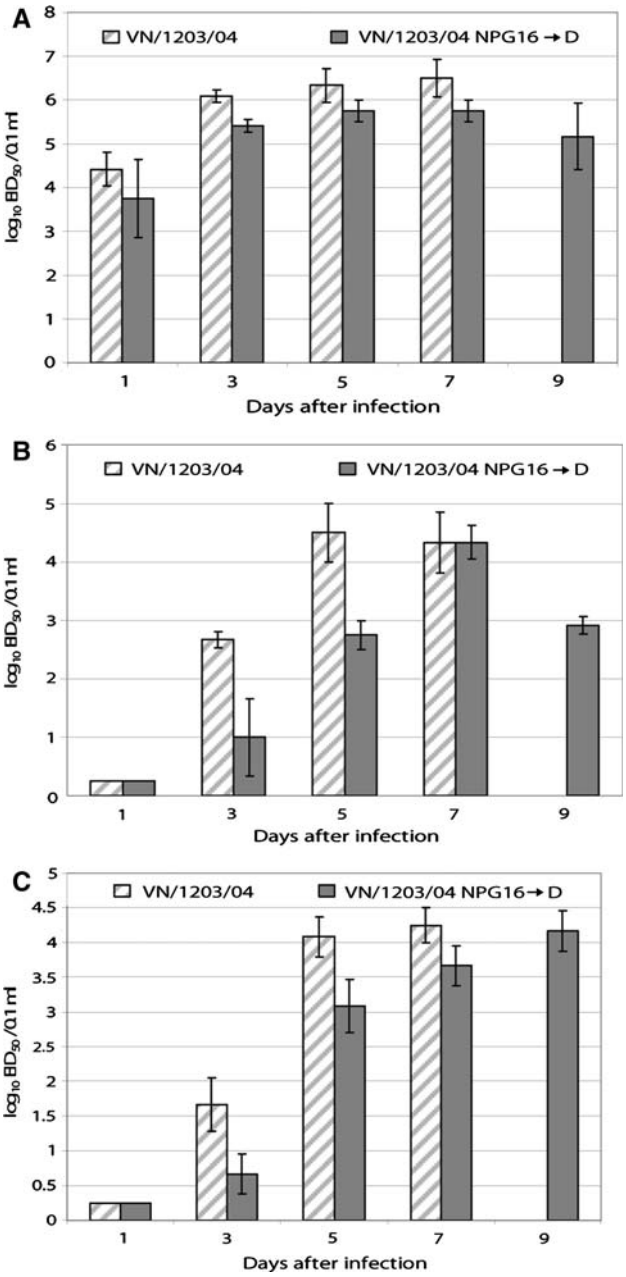

Fig. 4 Replication of recombinant avian H5N1 viruses in mouse lungs (**a**), brain (**b**), and spleen (**c**). Six-week-old BALB/c mice were inoculated intranasally with approximately 0.5×10^3 EID_{50} of virus. Each data point represents the mean ± SD virus titer (log_{10} EID_{50}/0.1 ml of 10% tissue homogenate) from three mice. Data from two experiments are presented

virus in mice, the difference in MLD_{50} between the wild-type and mutant viruses (VN/04 and VN/04 $NP_{G16\rightarrow D}$) was not as great as that seen between the human PR/8 and PR/8 $NP_{D16\rightarrow G}$ viruses (Tables 1, 2). However, the difference in MLD_{50} between VN/04 and VN/04 $NP_{G16\rightarrow D}$ was indeed significant ($P < 0.001$).

The N-terminal region of NP is involved in this protein's interaction with viral RNA and in the regulation of viral genome replication (see reviews [2, 16]). Amino acids K_7 and R_8 in NP play a key role in the interaction with karyopherin α, which regulates the transport of NP into the nuclei of infected cells [22]. The NP crystal structure indicates that this non-conventional nuclear localization signal identified at the N-terminus of NP can function as its NLS [25]. Recent data also showed that codon 16 is located within the region that is important for RNA packaging of segment 5 (NP) [6, 15], and that mutations in this part of the RNA molecule could affect the segment-packaging signal. Therefore, it seems predictable that modifications of the N-terminal region of NP will influence viral replication.

In this study we demonstrated that a single mutation in the N terminus of NP of the human PR/8 virus made this protein resistant to intracellular cleavage by caspases but significantly decreased the pathogenicity of the virus in mice. We also showed that NP that originated from an avian Teal/HK virus is incompatible with genes of the human PR/8 virus. The insertion of the human-like mutation, which made avian NP susceptible to cleavage by caspases, did not restore the compatibility of NP with gene segments of human virus, but attenuated the reassortant virus even more. The insertion of the caspase cleavage motif into NP of a highly pathogenic avian virus of the H5N1 subtype decreased its pathogenicity in mice.

On the basis of our overall results, we suggest that the presence of a caspase cleavage site in NP that originated from a human influenza virus as well as the absence of such a motif in the avian influenza NP is essential for the fitness of the virus. Alteration or substitution of this motif in NP resulted in attenuation of the human and avian influenza viruses in a mouse model. The mechanisms underlying this observation require further investigation. It is possible that caspase modifications of the NP could be important for viral protein–protein or protein–RNA interaction. Our results did not support the possibility that the conservation of caspase cleavage motif in the NP N-terminus is involved in host range determination of influenza A viruses. It is most interesting that differences among wild type and mutant viruses were significant and clearly recognizable in mice but not in cell culture experiments. This finding allows us to propose that the pathogenesis of disease caused by human and avian influenza viruses in mammals might be different.

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